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(54) Title: TSH RECEPTOR

(57) Abstract

A protein having the biological activity of a mammalian TSH receptor, and purified nucleic acid encoding such a protein.

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TSH RECEPTOR

Background of the Invention

This application is a continuation-in-part of Cone, U.S. Serial No. 404,899, filed September 8, 1989, entitled TSH RECEPTOR, hereby incorporated by reference herein.

This invention concerns nucleic acid encoding a mammalian thyroid stimulating hormone (TSH, also known as thyrotropin) receptor, and purified mammalian TSH receptors.

The TSH receptor is a protein believed to be involved in a human autoimmune disease termed "Graves'" disease. It is believed that antibodies against the TSH receptor are made in patients suffering from this disease. These auto-antibodies are currently detected by providing radiolabeled TSH, and detecting blocking of binding of the TSH to crude porcine membranes thought to include a TSH receptor.

Rees Smith et al. (Endocrine Reviews 9:106, 1988) describes the structure of a TSH receptor and predicts that clones of DNA encoding such receptors can be isolated by determination of the amino acid sequence of the TSH receptor and subsequent use of oligonucleotide probes to identify clones in a library. The receptor was only purified to about 0.001% purity (i.e., 10μg of TSH receptor in 1g of protein.

Summary of the Invention

Applicant has succeeded in isolating nucleic acid encoding at least two mammalian TSH receptors, and providing an expression system which enables production of large amounts of purified mammalian TSH receptor. Such purified receptor is useful in detection of auto-antibodies in patients suffering from Graves' disease or other malfunctions of the thyroid using simple antibody

assays, such as a competitive radioimmune assay or an ELISA test.

In a first aspect, the invention features purified nucleic acid encoding a protein having the immunological or biological activity of a mammalian TSH receptor. The purified nucleic acid can be purified cDNA, or a purified vector including that nucleic acid. In a related aspect, the invention features purified, e.g., recombinant, protein having the immunological or biological activity of a mammalian TSH receptor.

By "immunological activity" is meant the ability to selectively form an immune complex with auto-antibodies to the TSH receptor. By "purified" is meant that the nucleic acid or protein is provided separated from contaminating nucleic acid or other cell components, such as proteins and carbohydrates, with which the naturally occurring nucleic acid encoding the receptor occurs. Most preferably, the nucleic acid is provided as a homogeneous solution separated from all cell components, or is the major nucleic acid present in a preparation. More preferably, the nucleic acid is provided within a vector which is resident within a cell in a manner which allows expression of the nucleic acid to provide sufficient TSH receptor to be useful in this invention. By "recombinant" is meant that the protein is expressed from nucleic acid which has been manipulated by recombinant DNA methodology to place it in a vector or chromosome at a location in which it does not naturally occur. Preferably the purified protein is present at a purity of at least 10% of the total protein in a preparation, or even at 50% or 90% purity.

The biological activity of mammalian TSH receptor is that activity naturally associated with the TSH receptor of mammals, i.e., the ability of that protein to recognize and interact with TSH. It preferably includes

other biological activities of the TSH receptor such as activating adenylate cyclase, well known to those of ordinary skill in the art.

In preferred embodiments the TSH receptor is that receptor occurring in humans; the nucleic acid has a nucleotide sequence encoding an amino acid sequence identical to that of a naturally occurring mammalian TSH receptor, most preferably a human TSH receptor; or the nucleic acid encodes a protein having only conservative amino acid substitutions compared to a naturally occurring mammalian TSH receptor. Such conservative amino acid substitutions are well known to those skilled in the art and would include, for example, substitution of valine for glycine or leucine, substitution of a positively charged amino acid for another positively charged amino acid, or substitution of a negatively charged amino acid for another negatively charged amino acid. Such substitutions will not significantly affect the biological activity of the encoded TSH receptor; i.e., the biological activity of the substituted form will be at least 75% that of the naturally occurring form.

The proteins of the invention can be used in a method for detecting the presence of anti-TSH receptor antibodies in the serum of a patient. The method includes providing a purified TSH receptor as described above, and contacting that receptor with the serum. Reaction of the receptor with the serum is an indication of the presence of anti-TSH antibodies in that serum. This method may include any of many well known immunological procedures for detection of antibodies, such as ELISA, Western blot or competitive binding assays.

The present invention provides a sufficient amount of a mammalian TSH receptor to be useful for rapid

testing of patients for the presence of anti-TSH receptor antibodies. It also provides sufficient receptor protein to allow analysis of the sequence of the protein. Such analysis will aid determination of specific epitopes on that protein to allow design of small homologous peptides which will block the activity of autoimmune antibodies. Those peptides will thus block overstimulation of the thyroid in patients, such as those suffering from Graves' disease. The invention also provides the tools necessary to allow development of agonists or antagonists of TSH binding to a mammalian TSH receptor. These antagonists will be useful for preventing hyperthyroidism due to elevated levels of TSH.

In another aspect, the invention features a method for determining the presence of TSH in a sample. The method includes providing a mammalian cell having DNA encoding biologically active TSH receptor, the cell expressing TSH receptor from the DNA under assay conditions; contacting the cell with the sample to cause TSH within the sample to contact the cell; and measuring the level of intracellular cyclic adenosine monophosphate prior to and after the contacting step. An elevated level of cyclic adenosine monophosphate after the contacting step compared to prior to the contacting step is indicative of the presence of TSH within the cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a depiction of the nucleotide base sequence of the rat LH receptor probe.

Fig. 2 is a depiction of the nucleotide base sequence of the human LH receptor cDNA and the derived amino acid sequence.

Fig. 3 is a diagrammatic representation of the structure of the human LH receptor-encoding gene and the derived amino acid sequence.

Fig. 4 is a depiction of the nucleotide base sequence of degenerate oligonucleotide probes based on the human LH receptor DNA sequence.

Fig. 5 is a depiction of the partial nucleotide base sequence of the bovine and human TSH receptors. The boxed sequences indicate regions with possible sequence errors due to compression during sequence determination.

Fig. 6 is a depiction of the nucleotide base sequence and the derived amino acid sequence of the human TSH receptor cDNA.

Fig. 7 illustrates a darkfield photomicrograph (75x magnification) showing an autoradiographic signal (bright spots) produced by radiolabeled anti-sense transcript of human TSH receptor overlying a haematoxylin and eosin stained section of human thyroid.

Fig. 8 is a diagrammatic representation of the pATH3-hTSHR expression vector.

Fig. 9 illustrates a photograph of a polyacrylamide gel demonstrating the expression of the trp E-TSH receptor fusion protein (small arrow) in the absence (-) and presence (+) of indoleacetic acid. The small arrow indicates a protein of the size predicted for the fusion protein.

Fig. 10 is a graphical representation of the the level of intracellular cyclic adenosine monophosphate (cAMP) as a function of the concentration of applied hormone.

TSH Receptor

TSH receptors useful in this invention include any such receptor isolated from a mammal, or any protein having the biological activity of such a receptor. Such proteins will include proteins derived from naturally occurring TSH receptors having one or more of their amino acids modified conservatively as discussed above. Such modification may be by any standard procedure, for example, by recombinant DNA technology. Generally, such receptors will be expressed by recombinant DNA technology by isolating the gene encoding that receptor, and placing that gene within an expression vector, after removing any intronic DNA that may be deleterious to expression of the full length receptor protein. Such expression vectors will include bacterial, fungal, insect, and mammalian expression vectors which may be expressed within a bacterial, fungal, insect, or mammalian cell by techniques well known to those with ordinary skill in the art. Purified mammalian TSH receptor may be also be isolated by preparing antibodies to one of the above recombinant mammalian TSH receptors, and using those antibodies to immunoaffinity purify a naturally occurring TSH receptor. Generally, such a procedure is not preferred, since the yield of TSH receptor will be extremely small.

Once the desired TSH receptor protein is cloned, and its amino acid sequence determined, proteins having the biological activity of the receptor may be designed by standard procedure. For example, oligonucleotides may be synthesized by standard procedure, and inserted into any standard expression vector to cause expression of fragments of the naturally occurring TSH receptor. These fragments can be screened by standard procedure to determine whether they have the desired biological activity of the receptor protein. For example, it may be determined by affinity chromatography, Western blot

analysis, or some equivalent analysis, whether that synthetic peptide is able to bind with antibodies against a TSH receptor. Those fragments which can bind are useful in this invention. Similarly, the expressed TSH receptor, or that purified as described above, may be fragmented by use of enzymes, e.g., trypsin, which specifically cleaves the amino acid sequence into smaller fragments. These fragments may then be tested in much the same way as the synthetic peptide fragments to determine their usefulness in methods of this invention.

Below is presented one example of a mammalian TSH receptor-encoding gene, and expression of that gene within a vector to provide a purified mammalian TSH receptor. This example is not limiting to the invention and those skilled in the art will recognize many other mammalian TSH receptors can be isolated by identical procedures, or by use of the cloned DNA provided as deposits in the American Type Culture Collection (see below). The DNA in these deposits may be used to screen any existing or newly constructed library of mammalian DNA to determine the presence of clones encoding a part or all of a mammalian TSH receptor. Preferably such libraries will be constructed as cDNA libraries from RNA present in the thyroid of a mammal.

Example: Human and Bovine TSH Receptor

A 622 nucleotide fragment of the rat luteinizing hormone (LH) receptor gene was obtained from Deborah Segaloff of the Center for Biological Research at the Population Counsel, New York, New York, 10021, and from Peter Seeburg at the University of Heidelberg. This DNA fragment was used as a probe of a lambda-gt11 cDNA library constructed from RNA isolated from the thyroid of a patient suffering from Graves' disease. The nucleotide base sequence of this probe is shown in Fig. 1.

The cDNA library was constructed generally as follows. RNA of the thyroid was isolated using a standard guanidium/thiocyanate procedure and reverse transcribed using the method of Gubler and Hoffman. The resulting cDNA was size selected using a Sepharose G50 gel filtration column to select cDNA of greater than 1 kb in size. The cDNA was methylated with EcoRI methylase, linked to EcoRl linkers, and then treated with EcoRI. The resulting DNA was ligated to EcoRI treated lambda-gt11 DNA. The resulting lambda DNA was amplified in E. coli strain 1090.

The rat LH gene fragment was labeled with ^{32}P-dCTP and plates containing the lambda gt11 library screened on nitrocellulose filters at low stringency in 30% formamide, 1M NaCl, at 42°C. The filters were then washed at low stringency in 2 x SSC at 50°C.

Two classes of clones were detected, one class giving a strong reaction, and the other class a faint reaction with the probe. The strongly reacting plaques were purified three times using standard procedure, and four were determined to encode overlapping parts of the same gene by restriction endonuclease mapping, and DNA sequencing procedures. The 5' terminal 600 nucleotides of the gene showed high homology to the rat LH receptor. Further analysis determined that the cDNA encoded the full length human LH receptor protein with several introns remaining. The nucleotide base sequence is provided in Fig. 2. The amino acid sequence, molecular weight and isoelectric point of the encoded protein can be calculated by standard techniques from this sequence. The encoded protein has 90% homology in amino acid sequence to the rat LH receptor protein. The cDNA includes intronic DNA. RNA protection experiments, Northern analysis, and polymerase chain reaction experiments showed that the mRNA encoded by this clone is

expressed in the thyroid, testes, and ovary, as well as in Graves' thyroid, and in thyroid cell lines. The RNA is expressed in the thyroid but is incompletely spliced. Thus, this clone does not encode thyroid specific DNA.

In order to isolate clones encoding a TSH receptor, two degenerate oligonucleotide probes were constructed, one having homology to the transmembrane domain III of the above cloned human LH receptor DNA and the other having homology to the transmembrane domain VI of the LH receptor DNA. These domains and the location of the probes are shown in Fig. 3. These domains are separated by a distance of approximately 400 nucleotides in the cDNA. The oligonucleotides were synthesized and purified by standard procedure; their sequences are shown in Fig. 4.

Total RNA was isolated from a human Graves' thyroid, and from a bovine thyroid sample. Ten μg of total RNA from these two samples was separately reversed-transcribed using Moloney murine leukemia virus reverse transcriptase (commercially available). First strand cDNA was synthesized in a 50 μl reaction, and 5 μl of the resulting cDNA used in a polymerase chain reaction with the above synthetic oligonucleotides. This reaction had a total volume of 100 μl , including 5 μl of cDNA, 500 picomoles of each oligonucleotide, and the standard buffers and nucleotides described by Cetus Corporation (Emeryville, CA). This reaction was treated at 94°C for one minute in the presence of Taq DNA polymerase and then two minutes at 50°C and three minutes at 72°C. This cycle of heating and cooling between 50°C and 94°C was repeated thirty times. At this point, no amplification product could be observed. Five μl of the resulting reaction was removed and the procedure repeated. At this point, a DNA product was observed. No such product was observed in reactions using total RNA isolated from

osteosarcoma, testes, ovary, melanoma, or placenta. Thus, the DNA product appears to be thyroid specific. The resulting material was precipitated and resuspended by standard procedure, and digested with HindIII and EcoRI.

The EcoRI HindIII fragment was subcloned into the vector pBS⁻(Strategene, La Jolla, CA), and transformed into E. coli. The resulting vector was sequenced by Sanger dideoxy procedures. Both human and bovine cDNAs were sequenced and found to encode a protein having about 84% homology. Their tentative sequences are presented in Fig. 5. In contrast, the DNA had only about 68% homology with rat, porcine, and human LH receptor.

The fragments derived from the polymerase chain reaction were removed from the vector and labeled with ^{32}P. These fragments were then used as probes to screen the above described lambda-gt11 library at high stringency. The conditions were 50% formamide at 42°C in the presence of 1 M NaCl for 15-20 hours, and then washing of the nitrocellulose filters at 20-25°C in 2 x SSC for 15, minutes at 68°C in 1 x SSC for 45 minutes, and at 68°C in 0.1 x SSC for 45 minutes. Strongly hybridizing plaques were detected at a higher frequency than had been detected for the LH receptor clones. Twelve of these plaques were purified three times, purified DNA isolated from six, and analyzed by EcoRI restriction analysis. Four of these clones contained inserts of approximately 4.2 kb. These inserts were inserted into the pBS⁻ vector.

Northern blot analysis using the resulting clones showed that the DNA hybridized to RNA expressed only in the thyroid in both Graves' patients and the cold nodule sample, but not in the testes, ovary or other tissues. The DNA hybridized with an RNA of approximately 4.2 kb and thus appears to represent a full length clone of the

human TSH receptor. This RNA has a 3'-untranslated sequence of between 2 and 2.5 kb, and a 5'-untranslated sequence of approximately 50 bases. One clone, TR.12.6-1 (hTSH receptor), has been determined, by DNA sequencing, to contain a full length human TSH receptor cDNA (Fig. 6).

Further proof that the clone encoded human TSH receptor was provided by in situ hybridization histochemistry which demonstrated specific hybridization of anti-sense human TSH receptor probe to thyroid follicular cells which are known to respond to TSH (Fig. 7). Briefly, 8μm cryostat sections of normal appearing thyroid follicles were prepared for in situ hybridization. A 1 kb fragment of cDNA encoding the human TSH receptor was used to prepared ^{35}S labelled anti-sense transcript. Tissue sections were pre-treated with detergent and protease, and then incubated in hybridization buffer for 16 hours at 42°C with 3 x 10^5 CPM (specific activity approximately 10^8 cpm/μg) of probe as described (Hoefler et al., Histochem, J. 18:5597, 1986).

The above-described cDNA from human and bovine, or any other mammal may be expressed by standard procedures to provide large quantities of TSH receptor. For example, the above cDNA may be inserted into a trp E-fusion plasmid, e.g., pATH-1, 2, or 3, to form a stable hybrid protein with the Trp E protein. Alternatively, the cDNA may be inserted into a mammalian expression system such as a cytomegalovirus or retrovirus vector. Glycosylated protein will result when the DNA is expressed in the mammalian expression system.

Below is presented an example of a method to express TSH receptor. The amino terminal coding sequence of the human TSH receptor from a PstI site (nucleotide 346) to a HindIII site (nucleotide 1213) was ligated to

PstI/HindIII digested pATH3. The resulting plasmid, pATH3-HTSHR (Fig. 8), expresses a 66 kD fusion protein containing approximately 37 kD of the E. coli Trp E protein fused to 29 kD of the amino terminus of human TSH receptor protein. DH5α E. coli transformed with pATH3-hTSHR were grown in selective M9 media for 2 hours in either the absence or presence of 40 μg/μl indoleacetic acid, an inducer of the Trp E gene. Bacterial pellets were lysed in SDS loading buffer and 1/10th of the material was electrophoresed on a 10% polyacrylamide Laemmeli gel (Fig. 9).

Use

As discussed above, nucleic acid encoding TSH receptor may be used to express large quantities of TSH receptor. For example, high level expression is achieved with a Baculovirus vector pVL941, the E. coli vector paTH3, and the mammalian vector pLJ. Such protein is useful for detection of auto-antibodies found in Graves' patients. This allows determination of the state of the thyroid of those patients, and indicates the progress of that patient. This test may be performed in an ELISA format, for example, in a dipstick assay. The test might also take the form of a competitive binding assay employing radiolabeled TSH and TSH receptor. Such assays are extremely sensitive, and more readily performed than prior methods of detecting such antibodies.

The expressed protein is useful for defining the epitopes recognized by antibodies in Graves' patients. This analysis may be performed by standard procedure, for example, by expressing portions of the cloned DNA to provide partial TSH receptor fragments, or by fragmenting the expressed receptor protein as discussed above. Once the region recognized by such antibodies is defined, these fragments may be used in immunoassay procedures. In addition, definition of epitopes may be performed by.

manipulating the cloned genes using standard techniques of molecular biology to provide proteins in which one or more amino acids which may form a part of one or more epitopes of the protein is altered or deleted.

The protein or portions thereof is also useful as a therapeutic where it may be administered in a pharmaceutically acceptable compound at a sufficient dose to alleviate one or more symptoms of Grave's patients, or other patients suffering from thyroid malfunction. Generally, such administration will be at a level between one and one thousand micrograms per kilogram of patient.

Small peptides may be designed which will block the activity of auto-antibodies that act as TSH agonists, and thus block stimulation of the thyroid. Other small peptides may be designed which will block auto-antibodies that act as TSH antagonists. In addition, antagonists of TSH may be constructed which prevent binding of TSH to the TSH receptor and thus prevent elevated thyroid activity.

Assays for TSH

There follows two assays for TSH. The first assay technique is based upon the expression of TSH receptor within a cell which does not naturally contains such a receptor. This cell, when contacted with TSH, will increase expression of cyclic adenosine monophosphate, which can be detected as a measure of the amount of TSH in a sample.

In this assay, the human TSH receptor-encoding DNA is inserted with a mammalian retroviral vector pLJ at the *Bam*HI to *Sal*I sites. The resulting vector is then transfected into human 293 cells and clonal cell lines containing the vector isolated by selection in the presence of the antibiotic G418. Such transfection causes the cells to become responsive to TSH as measured by the activation of adenylate cyclase and accumulation

of cAMP following treatment with TSH. Thus, these cell lines provide a highly sensitive assay system for the hormone TSH. Cells in culture or cell membrane preparations may be exposed to the sample thought to contain TSH and the resulting adenylate cyclase activity quantitated and correlated with the cyclase activity from standard dilution curves of TSH in order to calculate the concentration of TSH in a sample. Concentrations as low as 1 ng/ml or even 0.1 ng/ml can be detected in this assay. This assay demonstrates that the TSH receptor encoded by the cDNA described above is biologically active and leads to specific TSH responsiveness in a previously unresponsive cell line. These cell lines are responsive not only to naturally occurring TSH but also to recombinant TSH.

Specifically, a retrovirus expression vector pLJ (Korman et al., Proc. Natl. Acad. Sci. USA 84:2150, 1987) containing the entire tr.12 cDNA sequence was transfected into human 293 cells and intracellular cAMP concentrations measured 60 hours later using a ^{3}H-cAMP displacement assay after treatment with hCG, hFSH, or hTSH. Referring to Fig. 10, 100 ng/ml of hFSH or hCG has little effect while the same amount of hTSH elevated intracellular cAMP over 6-fold. Half maximal intracellular concentrations of cAMP were obtained with approximately 60 picomolar hTSH. In several experiments, a 15-fold elevation of intracellular cAMP was induced by application of 100 ng/ml hTSH. Transfection of the retrovirus vector alone, with no hTSH-r insert, produced no elevation of intracellular cAMP over background in cells treated with 100 ng/ml TSH. Expression of the human LH/CG receptor was attempted using identical methods, however, no elevation of cAMP was seen after treatment with any of the glycoprotein hormones. This could result from any of

number of problems, including, for example, the deletion found in clone tr.13, or perhaps inefficient removal of the LH/CG-R introns in the non-gonadal 293 cell line.

The TSH receptor of this invention can be used to measure TSH by means of a competitive binding assay. In this assay TSH receptor, or a portion thereof capable of binding TSH, is immobilized on a support matrix. The immobilized receptor is incubated with excess TSH, which has been tagged with a radioactive or florescent label, long enough for the binding reaction to come to equilibrium. Unbound TSH is removed by a washing step, and the receptor is incubated with the test sample. Once this second binding step has come to equilibrium, the immobilized receptor is washed again. The amount of tagged TSH displace by TSH in the test sample then serves as a measure of the TSH present in the test sample. Other assays for TSH employing purified TSH receptor can be devised by those skilled in the art.

Deposits

The following DNA deposits were made on September 6, 1989, with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Maryland 28052 under the terms of the Budapest Treaty, where the deposits were given the following accession numbers:

Deposit	Accession No.
tr.12.6-1 (hTSH receptor)	40651
tr.13.t35 (hLH receptor)	40652

Applicant's assignee, New England Medical Center Hospitals, Inc., represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will

be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicants' assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Other embodiments are within the following claims

Claims

1. A purified nucleic acid encoding a protein having the biological activity of a mammalian TSH receptor.

2. Purified nucleic acid encoding a mammalian TSH receptor.

3. The purified nucleic acid of claim 1 or 2 wherein said mammalian TSH receptor is human TSH receptor.

4. Purified cDNA encoding a protein having the biological activity of a mammalian TSH receptor.

5. A purified vector comprising nucleic acid encoding a protein having the biological activity of a mammalian TSH receptor.

6. A cell comprising a vector comprising nucleic acid encoding a protein having the biological activity of a mammalian TSH receptor.

7. Purified protein having the biological activity of a mammalian TSH receptor.

8. A method for detecting the presence of anti-TSH receptor antibodies in the serum of a patient, comprising the steps of:

providing purified TSH receptor,

contacting said TSH receptor with the serum; and

detecting reaction of said TSH receptor with said serum as an indication of the presence of antibodies in the serum.

9. A method for determining the presence of TSH in a sample comprising the steps of:

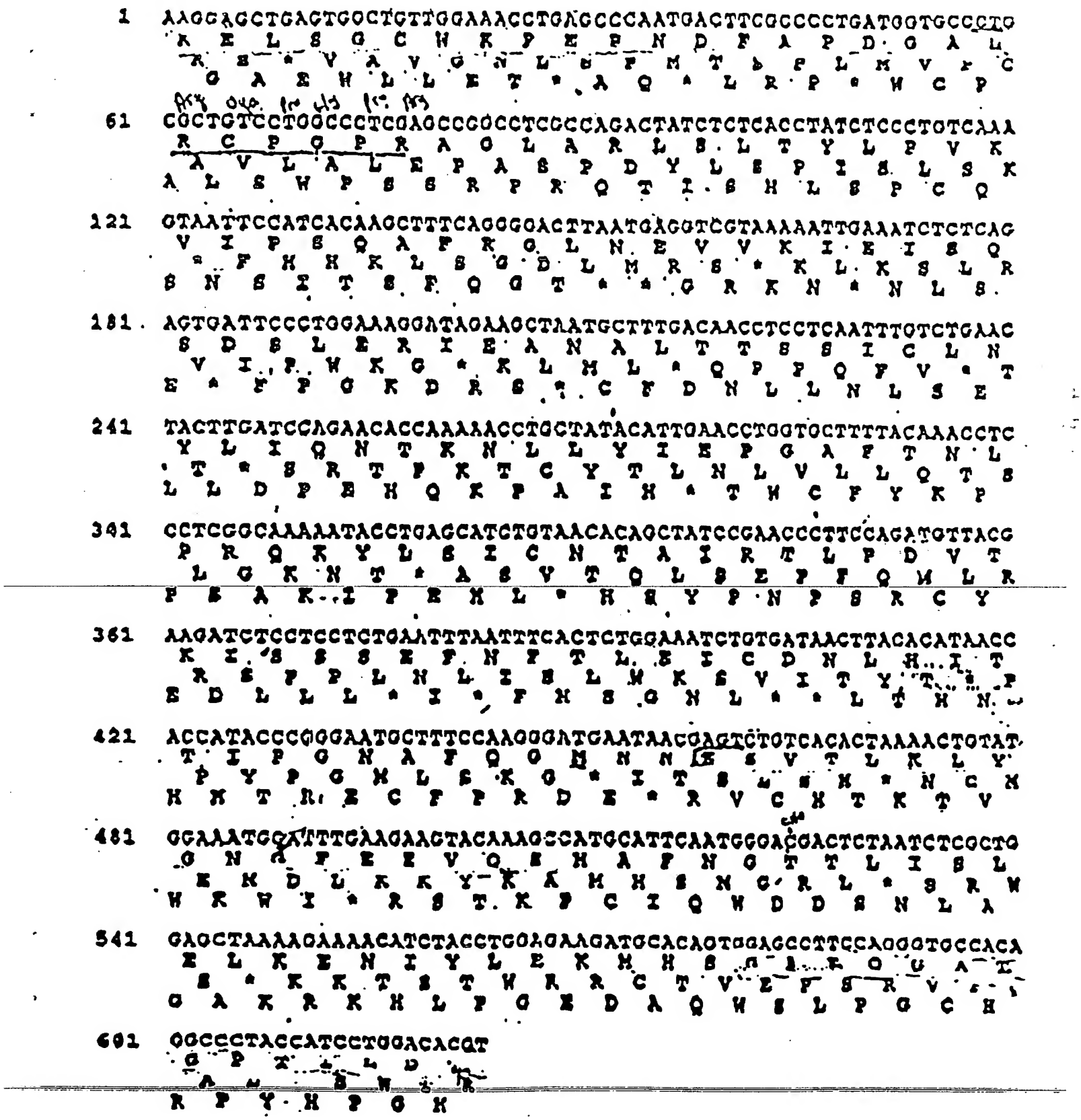
providing a mammalian cell comprising DNA encoding biologically active TSH receptor, said cell expressing TSH receptor from said DNA under assay conditions,

contacting said cell with said sample to cause TSH within said sample to contact said cell; and

measuring the level of intracellular cyclic adenosine monophosphate prior to and after said contacting step;

wherein an elevated level of cyclic adenosine monophosphate after said contacting step compared to prior to said contacting step is indicative of the presence of TSH within said cell.

FIG. 1



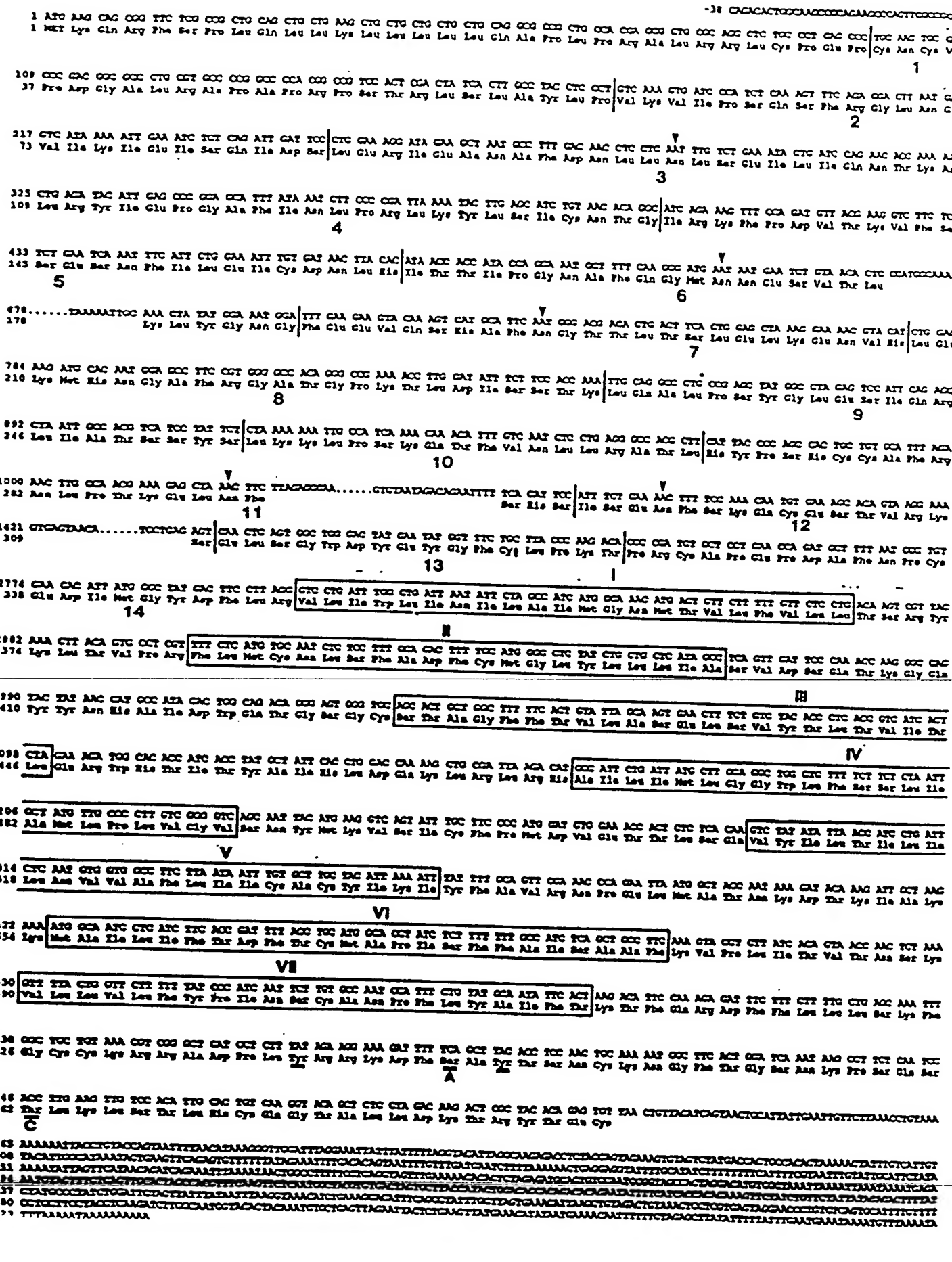


FIG. 3

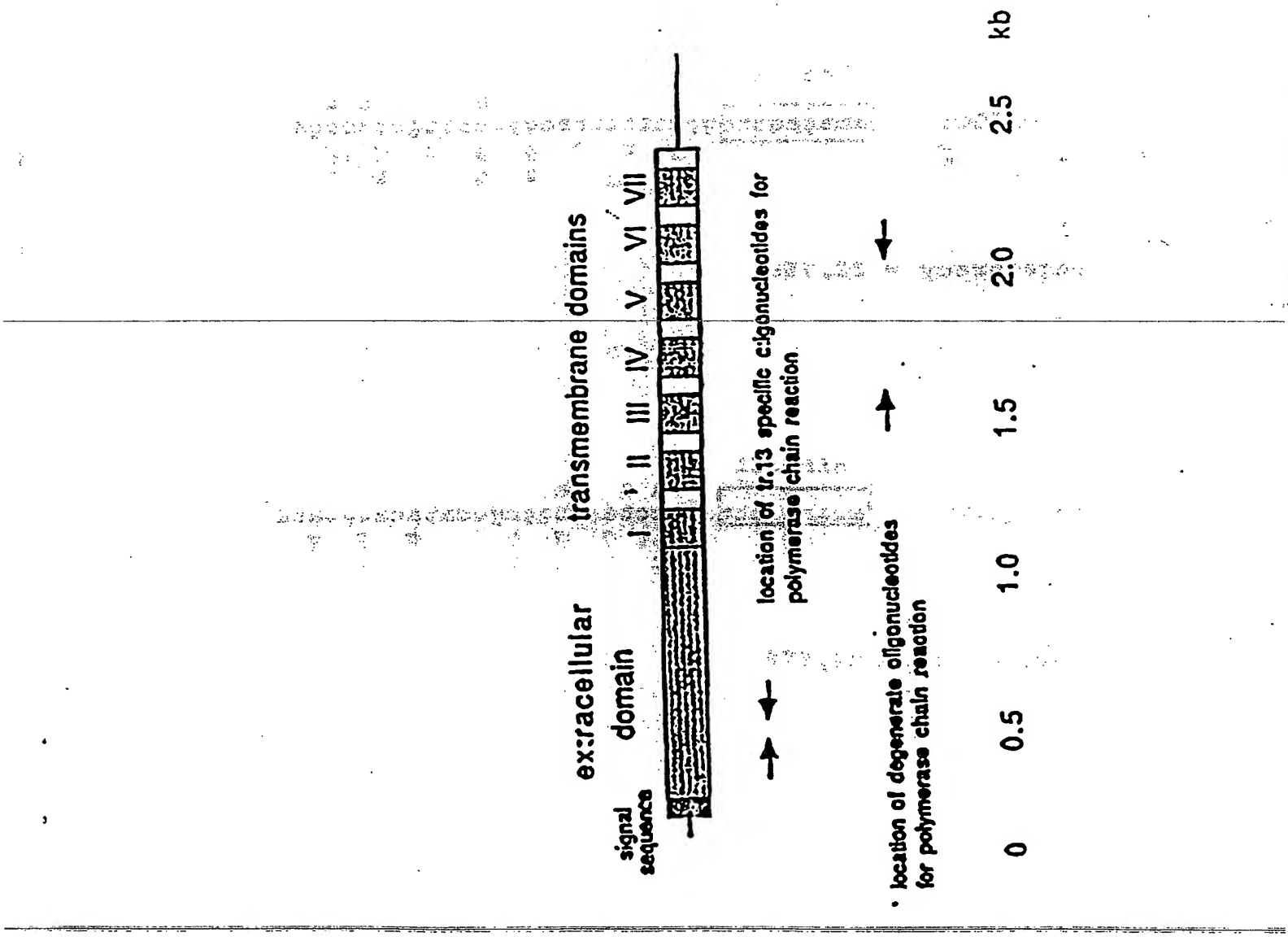

kb
2.5
2.0
1.5
1.0
0.5
0
extracellular domain
transmembrane domains
I II III IV V VI VII
signal sequence
location of 5'.13 specific oligonucleotides for polymerase chain reaction
location of degenerate oligonucleotides for polymerase chain reaction

FIG. 4

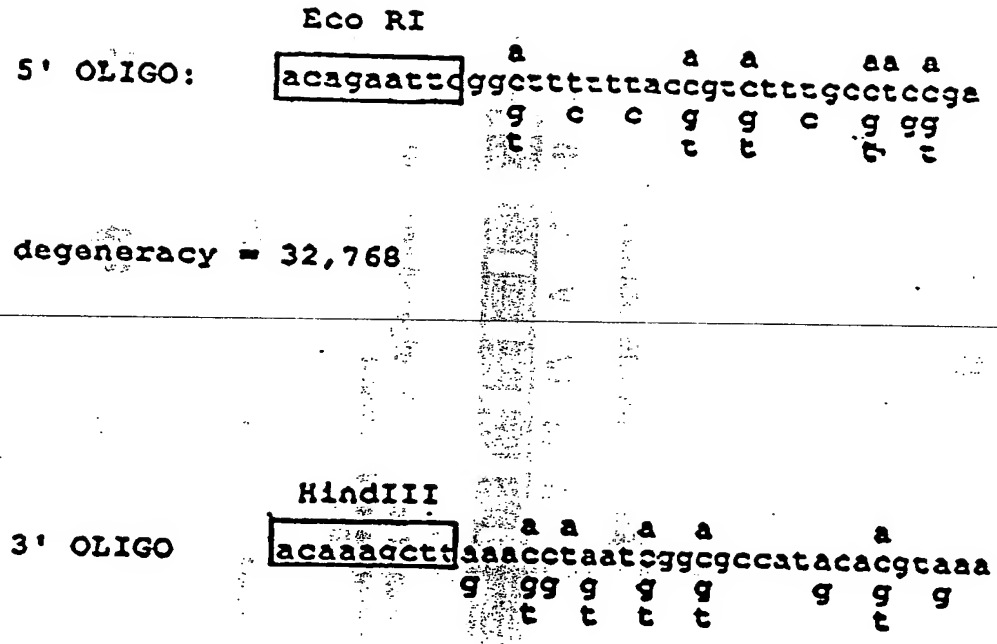

Eco RI
5' OLIGO:
acagaattc
degeneracy = 32,768
HindIII
3' OLIGO
acaaagctt
degeneracy = 24,576

FIG. 5 (sheet 1 of 2)

BOVINE PCR FRAGMENT (TSH RECEPTOR TRANSMEMBRANE DOMAINS 3-6)
PARTIAL SEQUENCE

```
GGG TTC TTC ACG GTG TTT GCG AGC GAG CTG TCT GTG TAC ACG CTG ACG GTC ATC
Gly Phe Phe Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Val Ile

                                        81                              108
ACC TTG GAG CGC TGG CAC GCC ATC ACC TTC GCC ATG CGC CTG GAC CGC AAG ATC
Thr Leu Glu Arg Trp His Ala Ile Thr Phe Ala MET Arg Leu Asp Arg Lys Ile

                                       135                              162
CGC CTC TGG CAC GCC TAC GTC ATC ATG CTG GGG GGC TGG GTT TGC TGC TTC CTG
Arg Leu Trp His Ala Tyr Val Ile MET Leu Gly Gly Trp Val Cys Cys Phe Leu

                                       189                              216
CTC GCC CTG CTC CCT TTC CTC CCA ATA AGC AGC TAT GCC AAC CTG CGC ATC TGC
Leu Ala Leu Leu Pro Phe Leu Pro Ile Ser Ser Tyr Ala Asn Leu Arg Ile Cys

CTG CCC ATG GAC ACC GAG
Leu Pro MET Asp Thr Glu
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HUMAN PCR FRAGMENT (TSH RECEPTOR TRANSMEMBRANE DOMAINS 3-6)

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                                        29                              56
GTT TTT CGT AGC GAG TTA TCG GTG TAT ACG CTG ACG GTC ATC ACC CTG GAG CGC
Val Phe Arg Ser Glu Leu Ser Val Tyr Thr Leu Thr Val Ile Thr Leu Glu Arg

                                        83                              110
TGG TAT GCC ATC ACC TTC GCC ATG CGC CTG GAC CGG AAG ATC CGC CTC AGG CAC
Trp Tyr Ala Ile Thr Phe Ala MET Arg Leu Asp Arg Lys Ile Arg Leu Arg His

                                       137                              164
GCA TGT CGG ATC ATG GTT GGG GGC TGG GTT TGC TGC TTC CTT CTC GCC CTG CTT
Ala Cys Arg Ile MET Val Gly Gly Trp Val Cys Cys Phe Leu Leu Ala Leu Leu

                                       191                              218
CCT TTG GTG GGA ATA AGT AGC TAT GCC AAA GTC AGT ATC TGC CTG CCC ATG GAC
Pro Leu Val Gly Ile Ser Ser Tyr Ala Lys Val Ser Ile Cys Leu Pro MET Asp

                                       245                              272
ACC GAG ACC CCT CTT GCT CTG GCA TAT ATT GTT TTT GTT CTG ACG GTC AAC ATA
Thr Glu Thr Pro Leu Ala Leu Ala Tyr Ile Val Phe Val Leu Thr Val Asn Ile
```

FIG. 5 (sheet 2 of 2)

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                                                    299                                                 326
GTT GGC TTC GTC ATC GTC TGC TGC TGT TAT GTG AAG ATC TAC ATC ACA GTC CGA
Val Gly Phe Val Ile Val Cys Cys Cys Tyr Val Lys Ile Tyr Ile Thr Val Arg

                                                    353                                                 380
AAT CCG CAC AAC CCA GGG GAC AAA GAT ACC AAA ATT GCC AAG AGG ATG GCT GTG
Asn Pro His Asn Pro Gly Asp Lys Asp Thr Lys Ile Ala Lys Arg MET Ala Val

                                                    407
TTG ATC TTC ACC GAC TTC ACG TGC ATG GCC CCC
Leu Ile Phe Thr Asp Phe Thr Cys MET Ala Pro
```

FIG. 6

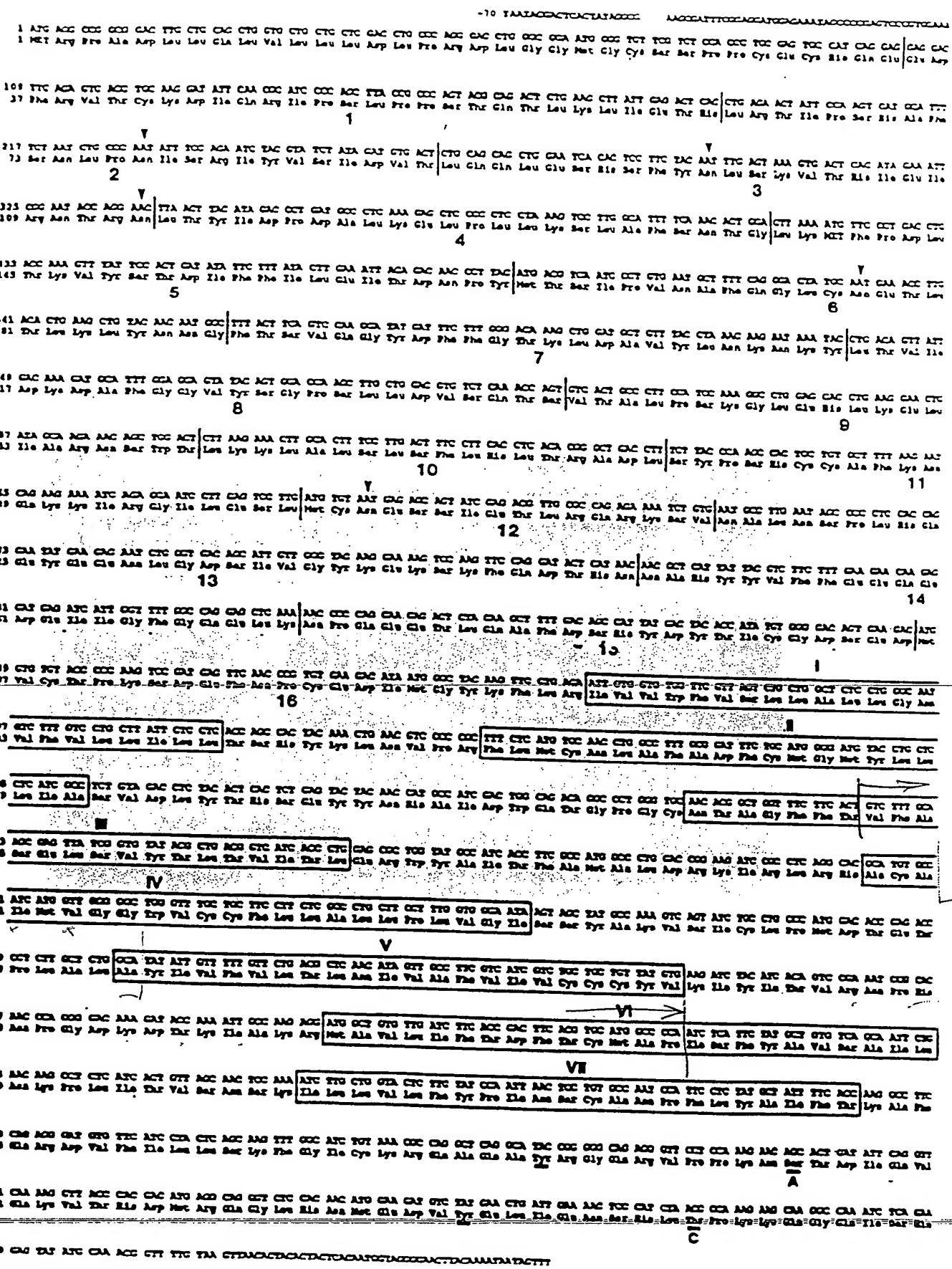


FIG. 7

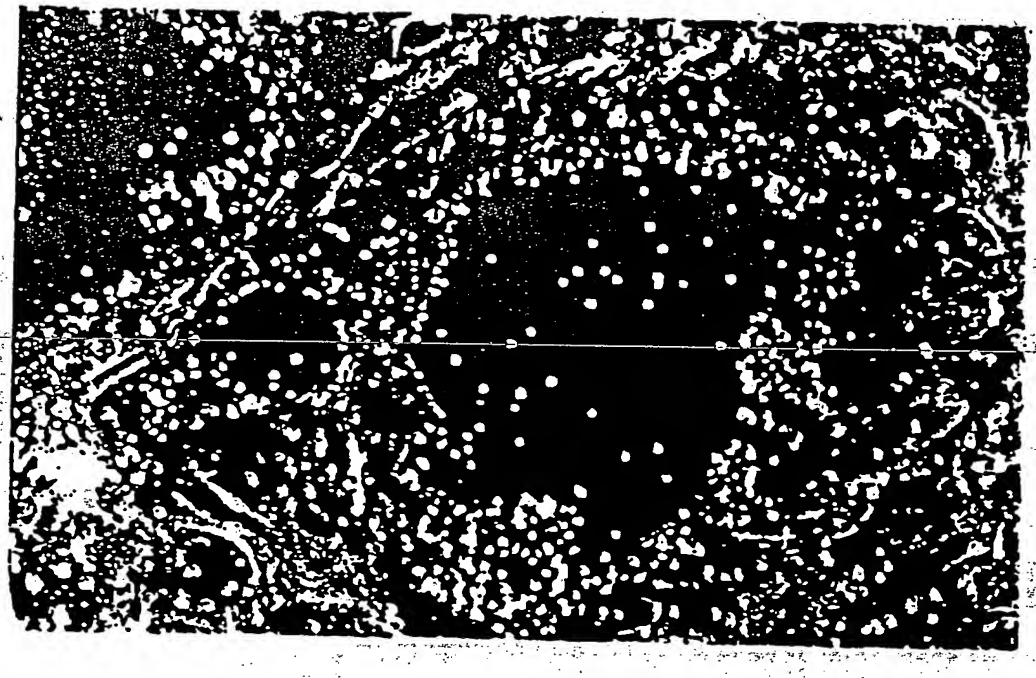


FIG. 8

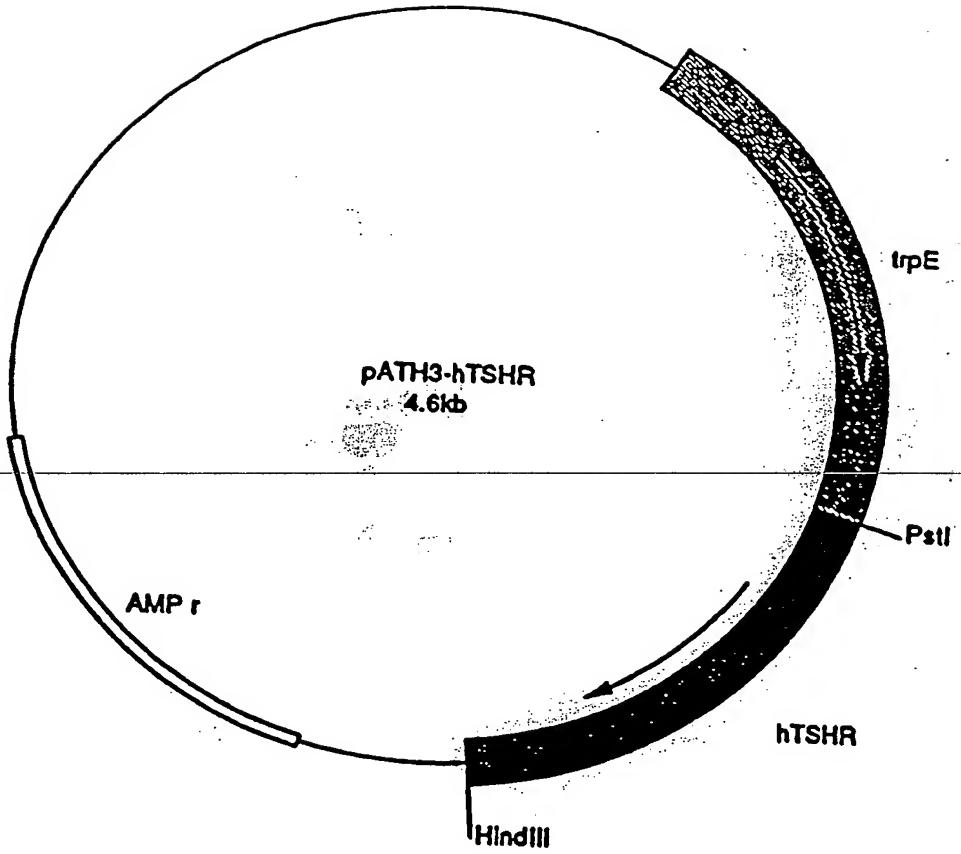


FIG. 9

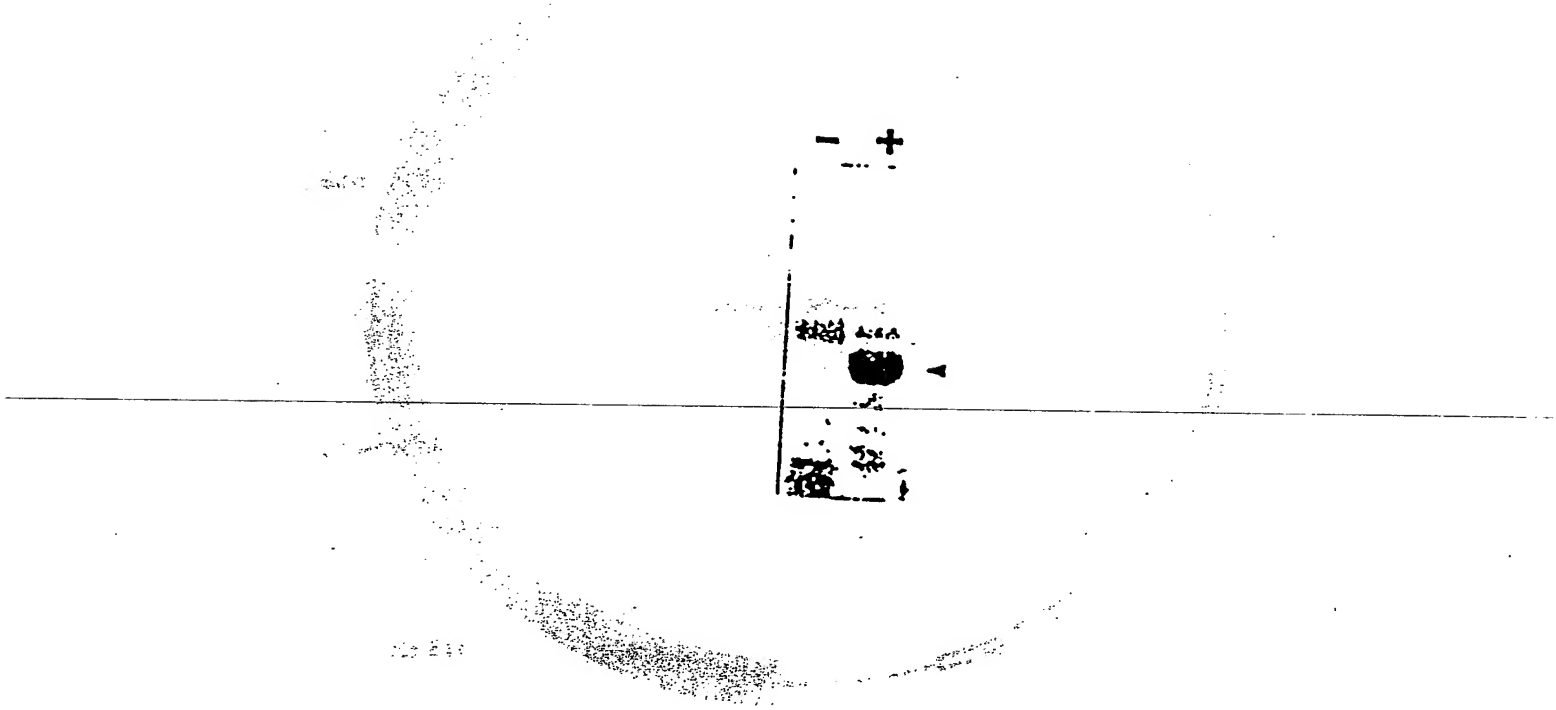
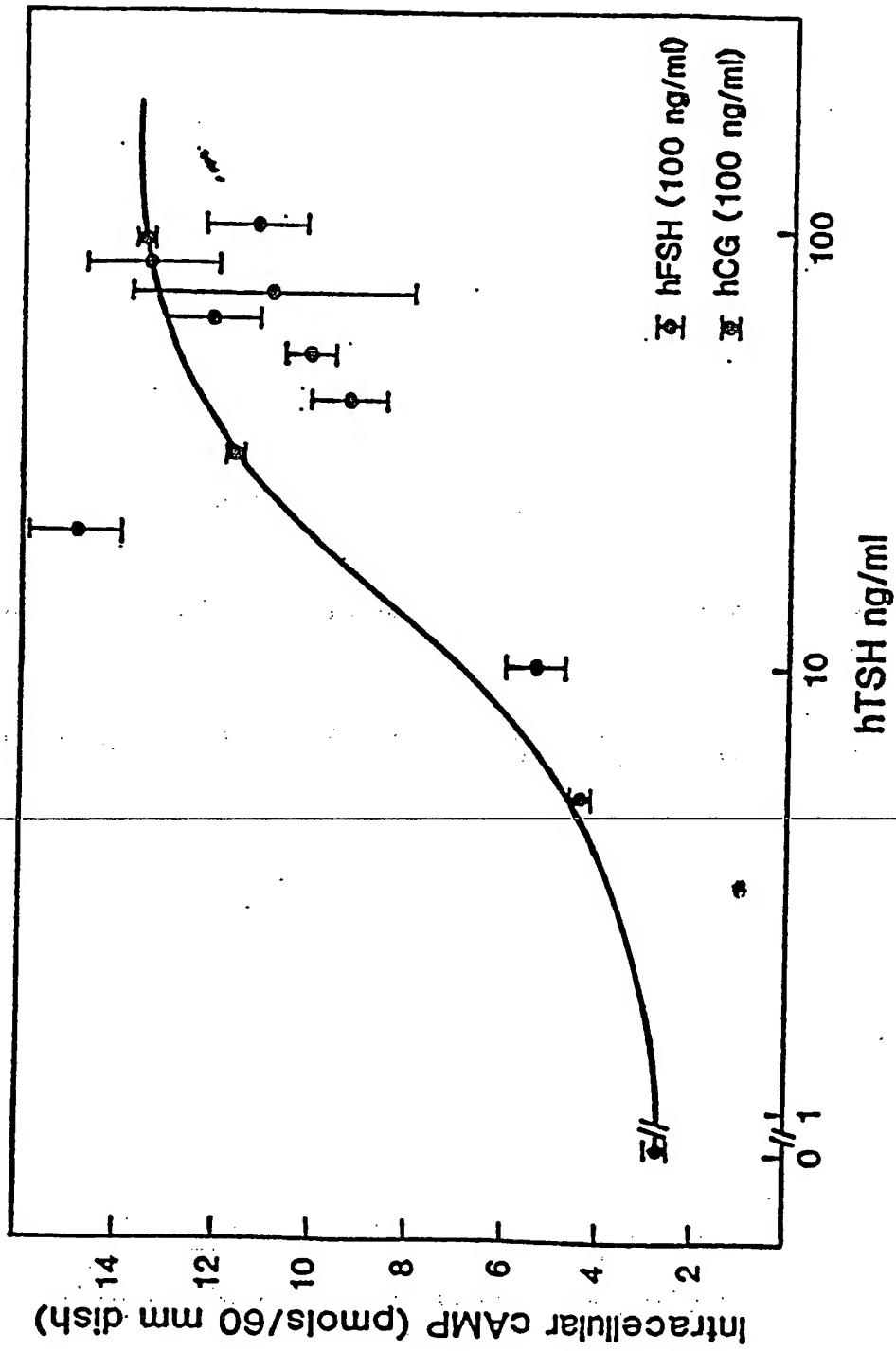


FIG. 10


hFSH (100 ng/ml)
hCG (100 ng/ml)
hTSH ng/ml
0
1
10
100
Intracellular cAMP (pmols/60 mm dish)
2
4
6
8
10
12
14

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05066

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07H 15/12; C12N 1/22; G01N 33/53; C07K 13/00
U.S. Class: 530/350; 436/500; 536/27; 435/320, 252.3

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/350; 436/500; 536/27; 435/320, 252.3

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	Biochemical Actions of Hormones, Vol. 12, issued 1985, Kohn et al., "The Thyrotopin Receptor," pages 457-512, see Table I and II.	7,8,9
X	Nature, Vol 330, issued 17 December 1987, de The et al., "A novel Steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma", pages 667-670, see entire document.	1-6

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
03 December 1990	15 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [11]
ISA/US	Shelly J. Guest

International Application No. PCT/US90/05066

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . ... . . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-6, drawn to DNA, vector, and host cell, classified in class 536, subclass 27, class 435, subclasses 320 and 252.3.

II. Claims 7 to 9, drawn to a protein and method of using, classified in c class 530 subclass 350 and class 436, subclass 500.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.